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ORIGINAL PAPER

Study on the Interaction between Florasulam and Bovine Serum Albumin

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Abstract In this paper, the interaction between florasulam (FU, 2′,6′,8-trifluoro-5-methoxy [Kragh-Hansen U, Molecular aspects of ligand binding to serum albumin. Pharmacol Rev 33 (1):17–53 1981; Carter DC and Ho JX, Structure of serum albumin. Adv Protein Chem 45:153–203 1994; He XM, and Carter DC, Atomic structure and chemistry of human serum albumin. Nature 358(6383):209–215 1992] triazolo [1,5-*c*] pyrimidine-2-sulfonanilide) and bovine serum albumin (BSA) was investigated by fluorescence, ultraviolet absorption (UV) and Far-UV circular dichroism (CD) spectrometries. A strong fluorescence quenching was observed and the quenching mechanism was considered as static quenching. The binding constant of FU with BSA at 299 and 309 K were obtained as 1.5×10^4 and 7.1×10^3 l mol^{-1}, respectively. There was one binding site between FU and BSA. The thermodynamic parameters enthalpy change (ΔH) and entropy change (ΔS) were calculated as −57.89 kJ mol^{-1} and −113.6 J mol^{-1} K^{-1}, respectively, which indicated that the acting force between FU and BSA was mainly hydrogen bond and Van der Waals force. According to the Förster non-radiation energy transfer theory, the average binding distance between donor (BSA) and acceptor (FU) was obtained (r=1.59 nm). The investigations of the UV/Vis and CD spectra of the system showed that the conformation of BSA was changed in presence of FU.

Keywords Florasulam · Bovine serum albumin · Fluorescence quenching · Förster non-radiative energy transfer

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Introduction

Serum albumins such as bovine serum albumin and human serum albumin are plasma proteins contributing considerably to physiological functions [1, 2], which play important roles in the transport, distribution and metabolism of many exogenous ligands, including fatty acids, amino acids, drugs and pharmaceuticals [3–6]. In recent years, investigations of the interactions of small molecules, such as dye, drugs and toxic chemicals with proteins [7–10] have been the focus of research field in life sciences, chemistry and clinical medicine. The interactions of small molecules with serum albumin can elucidate the properties of small molecule–protein complex, and it may provide useful information of the structural features of small molecules. BSA is considered as an in vitro model for studying drug–protein interaction since BSA is a single-chain 582 amino acid globular nonglycoprotein cross-linked with 17 cystine residues, and its structure is similar to human serum albumin, HSA in 76% [4, 11].

Pesticide, including herbicide, is a kind of chemical reagent which is widely used to kill pests such as vermin, rats, weed, and so on. The use of pesticide is of great benefit to human being, but it can also contaminate the entironment. As we know, only 10 percent of pesticide can be utilized, and the residues volatilize to the air, flow to rivers and congregate in the soil, causing to the contamination of farm, cattle breeding and aquatic products. And

pesticide can be transferred to the body of human being through the food cycle, which can endanger the health of human being. Florasulam is the fifth triazolopyrimidine sulfonanilide post-emergence broadleaf herbicide for use in wheat (early postemergence) empoldered in the middle of 1990s [12]. It has a good application foreground because of its high biological activity and low doses, whereas, as a kind of herbicide, florasulam has some toxicity via the oral, dermal and inhalation routes of exposure. Its structure is shown in Scheme 1.

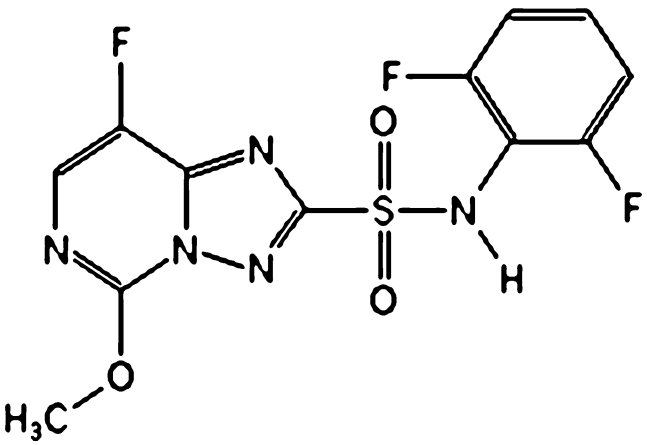
The investigation of the interactions between FU and proteins is in favor of reasonable utilization of FU. In this paper, the details of the binding modes of FU to BSA were studied through the binding strength, position and alteration of the conformation of protein using fluorescence, UV absorption and Far-UV CD spectrometries.

Materials and methods

Reagents

A stock solution of BSA (1.00×10^{-4} g ml^{-1}) was prepared by dissolving 0.0100 g BSA (Shanghai Boao Biochemical Technology, China) in 100 ml double-distilled water, stored at 0–4°C. A stock solution of FU (1.00×10^{-3} mol l^{-1}) was prepared by dissolving 0.0359 g FU (Shandong Pesticide Research Institute) in 2 ml 0.1 mol l^{-1} NaOH, then diluted with water in 100 ml volumetric flask. A 9% NaCl solution was prepared. A 0.05 mol l^{-1} Tris–HCl buffer solution was prepared by dissolving appropriate Tris in water and adjusting the pH to 7.40 with HCl. A 1.0 mol l^{-1} KI solution was prepared.

Unless otherwise noted, all reagents and solvents used in this study were analytical grade.



Scheme 1 Molecular structure of florasulam

Apparatus

All Fluorescence spectra were recorded on a LS-55 spectrofluorimeter (PE) in a 1 cm quartz cell. The ultraviolet absorption spectra were performed with a UV-4100 spectrophotometer (Hitachi, Japan) equipped with 1 cm quartz cells. Far-UV circular dichroism (CD) spectroscopy was carried out on a JASCO J-810S sepctropotarimeter (Japan). All pH measurements were made with a Delta 320-s pH meter (Mettler Toledo, Shanghai).

Procedures and spectral measurements

Solutions were added to a 25-ml test tube in the following order: 1.0 ml of 9% NaCl, 1.0 ml of 0.05 mol l^{-1} Tris–HCl buffer, 1.0 ml of 1.0×10^{-4} g ml^{-1} BSA and appropriate amount of FU, and the mixture was diluted to 10 ml with water.

The fluorescence spectra were recorded in the range of 300–420 nm with excitation and emission wavelengths at 280 and 347 nm, respectively. The excitation and emission slits were both 10 nm, with a scan speed of 500 nm min^{-1}. The UV/Vis and Far-UV CD spectra were recorded in the range of 190–240 nm and 200–260 nm, respectively.

Result and discussion

The fluorescence quenching and the binding constant studies

From the fluorescence spectra, we could obtain some information on the binding of small molecules to proteins [13, 14], such as the binding mechanism, binding constant, binding site and intermolecular distance. The fluorescence spectra of BSA in the presence of different FU concentrations were recorded in the range of 300–420 nm with excitation wavelength of 280 nm (Fig. 1). From Fig.1, it could be seen that BSA had a strong fluorescence emission peak at 347 nm, whereas FU had no intrinsic fluorescence. The fluorescence intensity of BSA could be quenched by FU, without a change in the position of emission maximum, which indicated that there was interaction between FU and BSA.

Fluorescence quenching is usually classified as dynamic quenching and static quenching, which can often be distinguished by their differing dependence on temperature. Since higher temperatures result in larger diffusion coefficients for dynamic quenching, and the quenching constant is expected to increase with increasing temperature. In contrast, a higher temperature may bring about the decrease in the stability of the complexes, resulting in lower quenching constant for the static quenching.

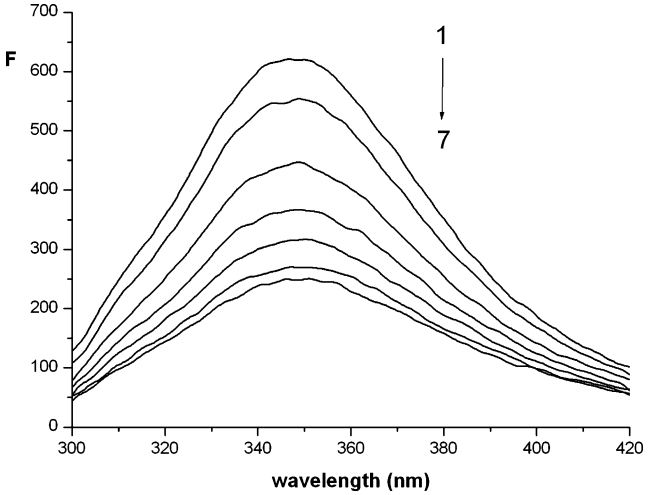


Fig. 1 The fluorescence quenching spectra of BSA by FU (λ_{em}= 280 nm). Conditions: BSA=1.0×10^{-5} g ml^{-1}; FU=0 (*1*); 1.0×10^{-5} mol l^{-1} (*2*); 3.0×10^{-5} mol l^{-1} (*3*); 5.0×10^{-5} mol l^{-1} (*4*); 7.0×10^{-5} mol l^{-1} (*5*); 9.0×10^{-5} mol l^{-1} (*6*); 1.0×10^{-4} mol l^{-1} (*7*); NaCl= 0.9%; Tris–HCl buffer=0.005 mol l^{-1} (pH 7.40)

It is assumed that the fluorescence quenching of BSA induced by FU is a dynamic quenching process, and the fluorescence quenching is described by using the Stern–Volmer equation [15, 16]:

$$F_0/F = 1 + K_{SV}[Q] = 1 + K_q\tau_0[Q] \quad (1)$$

Where F and F_0 are the fluorescence intensities with and without the presence of FU, respectively, K_{SV} is the Stern–Volmer quenching constant, K_q is the bimolecular quenching rate constant, τ_0 is the lifetime of the BSA in the absence of FU, and $[Q]$ is the concentration of FU. Hence, Eq. 1 is applied to determine K_{SV} by linear regression of a curve of F_0/F versus [FU].

The Stern–Volmer curves of F_0/F versus [FU] at different temperatures were plotted, as shown in Fig. 2. The plots showed that within the investigated concentrations, the results exhibited a good linear relationship. The Stern–Volmer quenching constants K_{SV} calculated from the slope of the regression curves were 1.5×10^4 l mol^{-1} at 299 K and 7.1×10^3 l mol^{-1}at 309 K, respectively. From Eq. 1, we knew $K_{SV}=K_q\tau_0$. Since the typical fluorescence lifetime of biopolymers was about 10^{-8} s, thus K_q were obtained as 1.5×10^{12} l mol^{-1} s^{-1} at 299 K and 7.1×10^{11} l mol^{-1} s^{-1}at 309 K, respectively. These values were much larger than the limiting diffusion rate constant of the biomolecule (2.0×10^{10} l mol^{-1} s^{-1}). So it was implied that the static quenching was dominant in the system.

The number of binding sites

For the static quenching interaction, if it is assumed that there are similar and independent binding sites in the biomolecule, the binding constant and the number of binding sites can be got from the double logarithm regression curve [17, 18]:

$$\lg((F_0 - F)/F) = \lg K + n\lg[Q] \quad (2)$$

Where K is the binding constant for quencher–protein interaction, n is the number of binding sites per BSA, and F_0, F, $[Q]$ has the same meaning as in Eq. 1.

The values of K and n could be calculated from the intercept and slope by plotting log $(F_0-F)/F$ against log [FU] (Fig. 3). The double logarithm regression curve of BSA–FU was linear in the whole measured concentration range of FU, and the binding constant K and binding sites n of FU to BSA were 1.5×10^4 l mol^{-1} and 0.998, respectively. Here the value of n almost equals to 1 indicating that there was one binding site in BSA for FU.

The binding site

KI is a fluorescence quencher, which can quench the fluorescence of the tryptophan residues only on the surface of the protein, but can not enter the inner portion of the protein to quench the fluorescence of tryptophan. Hence I^- can be used to measure the fraction of tryptophan residues on the surface of BSA [1, 19].

I^- quenching data were analyzed according to the modified Stern–Volmer equation [20]:

$$F_0/\Delta F = 1/(f_a K_{SV}[Q]) + 1/f_a \quad (3)$$

Where K_{SV} is the Stern–Volmer fluorescence quenching constant, $\Delta F=F_0-F$ is the difference of the fluorescence intensity of BSA with and without I^-, f_a is the fraction of residues that can be quenched by I^-, and $[Q]$ is the concentration of I^-.

The values of f_a and K_{SV}, calculated from the intercepts and slopes of the plots of $F_0/\Delta F$ vs. $1/[I^-]$ (Fig. 4), were 40.6% , 51.27 l mol^{-1} in the system of BSA–I^- and 89.4%,

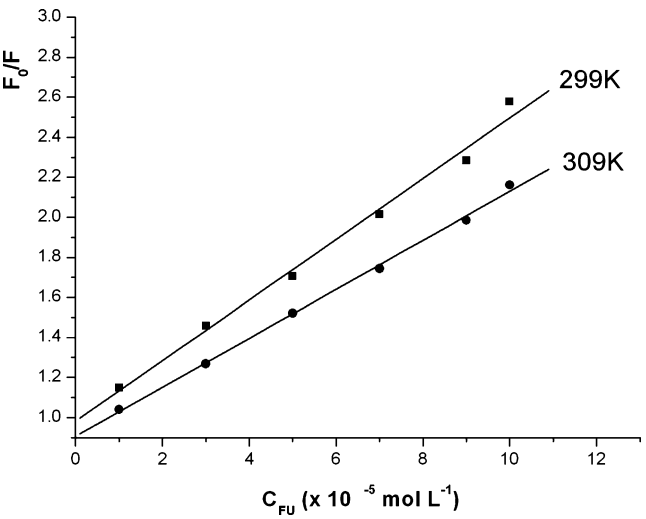


Fig. 2 The Stern–Volmer curves

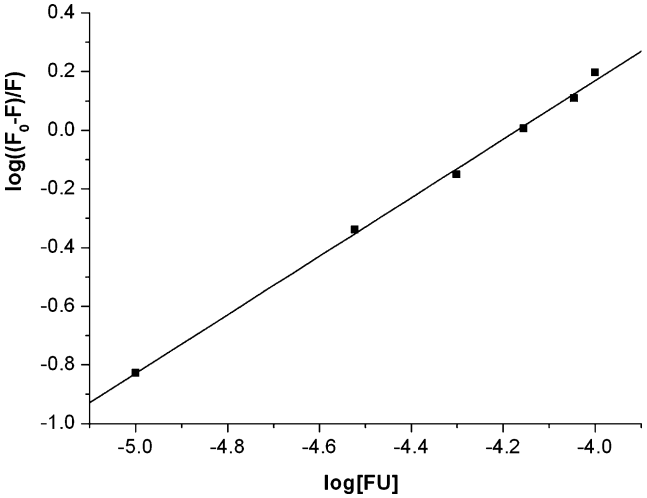


Fig. 3 Plot of log $(F_0-F)/F$ vs. log [FU]

3.04 l mol^{-1} in the system of FU–BSA–I$^-$, respectively. From the result it could be seen that the fraction of tryptophan residues on the surface of BSA in presence of FU was higher than that in absence of FU. There were two possibilities causing this phenomenon: one was the increase of tryptophan residues number on the surface absolutely, the other was the decrease of tryptophan residues number in the interior, leading to relative increase of tryptophan residues number on the surface. BSA was characterized by two tryptophan residues: Trp-212 was thought to be located within a hydrophobic binding pocket of the protein (in subdomain IIA) and Trp-134 was considered to be located on the surface of the albumin molecule. As it was known, FU was a triazolopyrimidine sulfonanilide molecule; it was difficult for FU to interact with polar tryptophan residues on the surface of BSA. So it was considered that FU entered the hydrophobic cavity of BSA and interacted with Trp-212, leading to the decrease of tryptophan residues number in the interior and relative increase of tryptophan residues number on the surface. This supposition was consistent with the aftermentioned conclusion that the hydrogen bond and Vander Waals force played major roles in the binding reaction of FU with BSA. In addition, the value of K_{SV} decreased as FU added. Maybe the reason was that the interaction between FU and BSA changed the conformation of BSA, which weakened the interaction between I$^-$ and BSA.

The acting force between FU and BSA

The acting forces between pharmaceutical and biomolecule include hydrogen bond, Vander Waals force, electrostatic force and hydrophobic interaction force and so on [21–23]. The temperature dependence of the binding constant allows us to estimate the binding enthalpy change according to the van't Hoff relationship [24]: If the enthalpy change (ΔH) does not vary significantly over the temperature range studied, the values of ΔH, ΔS and free energy change (ΔG) at different temperature were calculated from Eqs. 4 and 5.

$$\ln\left(K_2/K_1\right) = {}^{\Delta H}/_{R(1/T_1 - 1/T_2)} \tag{4}$$

$$\Delta\Delta G = \Delta H - T\Delta S = -RT\ln K \tag{5}$$

Where K_1 is the binding constant at the corresponding absolute temperature T_1, which can be calculated by Eq. 2, R is the universal gas constant. According to Eq. 2, the values of K_1 were 1.5×10^4 and 7.1×10^3 l mol^{-1} at 299 and 309 K, respectively. Thus, ΔG, ΔH and ΔS were obtained in the binding reaction between FU and BSA as −23.92 kJ mol^{-1}, −57.89 kJ mol^{-1} and −113.6 J mol^{-1} K^{-1}, respectively. The negative value of ΔG meant that the binding process was spontaneous. The negative values of both ΔH and ΔS indicated that the hydrogen bond and Vander Waals force played major roles in the binding reaction of FU with BSA.

Energy transfer between FU and BSA

The fluorescence studies prove that BSA can form a complex with FU, and the distance between tryptophan residues of BSA and FU molecules may be calculated according to the Förster non-radiative energy transfer theory [25, 26]. The energy transfer efficiency (E) is related not only to the distance (r) between the acceptor and donor, but also to the critical distance (R_0), which is the distance

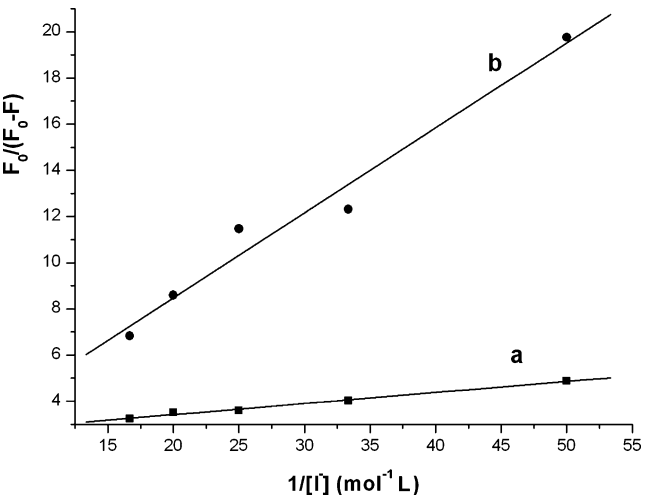


Fig. 4 Effect of the concentration of I$^-$ on the fluorescence of BSA. **a** BSA–I$^-$, **b** FU–BSA–I$^-$. Conditions: NaCl=0.9%; Tris–HCl buffer= 0.005 mol l^{-1} (pH 7.40); BSA=1.0×10^{-5} g ml^{-1}; FU=5.0×10^{-5} mol l^{-1}; I$^-$=0, 0.02, 0.03, 0.04, 0.05, 0.06 mol l^{-1}

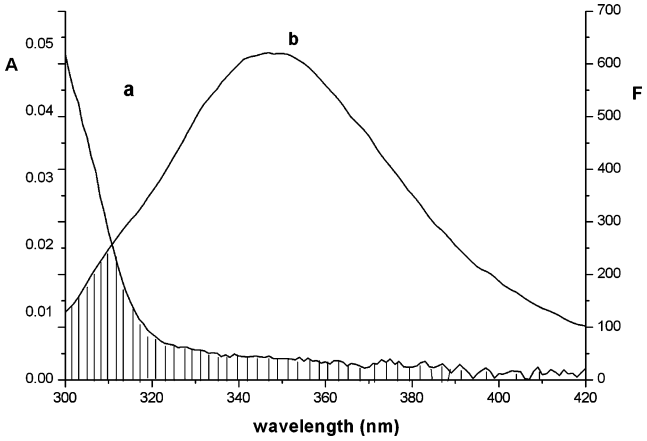


Fig. 5 Spectral overlap of FU absorption (*curve a*) with BSA fluorescence emission (*curve b*). **a** FU, **b** BSA. Conditions: BSA=1.0×10^{-4} g ml^{-1}, FU=5.0×10^{-5} mol l^{-1}

between the acceptor and donor when the energy transfer efficiency is 50%.

$$E = R_0^6/(R_0^6 + r^6) \tag{6}$$

$$R_0^6 = 8.8 \times 10^{-25} K^2 N^{-4} J\Phi \tag{7}$$

In Eq. 7, K^2 is a factor that describes the relative orientation in space of the transition dipoles of the donor and acceptor, N is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor in the absence of acceptor. The overlap integral J, which expresses the degree of spectral overlap between the donor emission and the acceptor absorption, can be written in the alternative form on the wavelength (λ) scale.

$$J = \sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda \Big/ \sum F(\lambda)\Delta\lambda \tag{8}$$

Where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor in wavelength λ and is dimensionless, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor in wavelength λ. Then the energy transfer efficiency can be determined from the following equation:

$$E = 1 - F/F_0 \tag{9}$$

The spectral overlap of FU absorption (curve a) with BSA fluorescence emission (curve b) was shown in Fig. 5. According to the degree of the spectral overlap, the value of J could be evaluated, which was 1.24×10^{-16} cm^6 l mol^{-1}. Thus, we could obtain that R_0=1.50 nm from Eq. 7 using K^2=2/3, N=1.336, Φ=0.118, the energy transfer efficiency E=0.41 from Eq. 9, and the distance between FU and tryptophan residue in BSA r=1.59 nm.

The conformation change of BSA

From the ultraviolet absorption (UV) spectra shown in Fig. 6, it could be seen that the absorption peak of BSA at 200 nm decreased and moved to longer wavelength when FU was added into BSA, which indicated that there was interaction between them. It was well known that the peak at about 200 nm could reflect the framework conformation of the protein [27]. The movement of the absorption peak indicated that the polarity of the microenvironment around the BSA was changed. The above phenomena indicated that the binding may lead to conformation change of BSA and the major conformation was reconstructed into the α-helix, which was an unfolding process.

Far-UV circular dichroism (CD) spectroscopy could give quantitative information in details on the conformation changes of the protein [28, 29]. Figure 7 showed the CD spectra of BSA with and without FU. It could be seen that the CD spectra of FU–BSA exhibited a larger molar

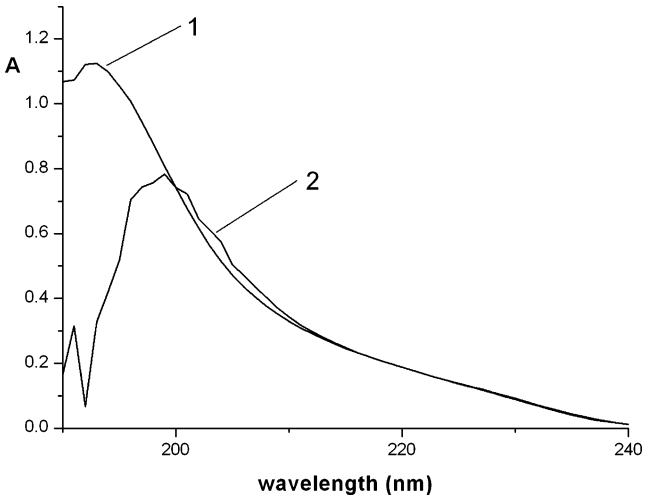


Fig. 6 Absorption spectra of FU–BSA system: BSA (1), FU–BSA (2). Conditions: BSA=2.0×10^{-5} g ml^{-1}, FU=5.0×10^{-5} mol l^{-1}

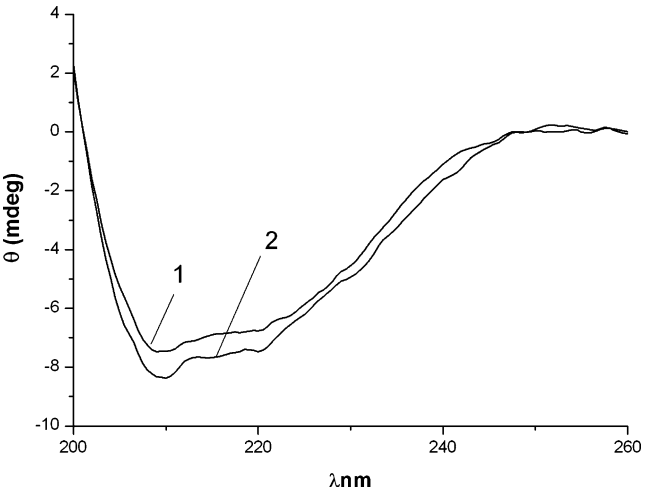


Fig. 7 Far-UV CD spectroscopy of FU–BSA system: BSA (*1*), FU–BSA (*2*). Conditions: BSA=5.0×10^{-5} g ml^{-1}, FU=1.0×10^{-4} mol l^{-1}

ellipticity than that of BSA at 220 and 209 nm, which indicated an increase of the α-helix content in BSA structure. The α-helix content increased to 37.8% from about 27.7% of the native BSA. At the same time, the β-sheet content decreased from 34.8% to 20.0%. The results agreed with that of ultraviolet absorption spectroscopy.

It could also be seen that the experimental conclusion obtained by UV and CD spectra agreed with the foregoing supposition in binding site experiment, i.e., the conformation of BSA was changed by the interaction between FU and BSA.

Conclusion

This paper investigated the interaction between protein and FU using fluorescence, UV and CD techniques. The results showed that the fluorescence of BSA was quenched by FU through static quenching. The binding constant of FU with BSA at 299 and 309 K were calculated as 1.5×10^4 and 7.1×10^3 l mol^{-1}, respectively. The acting forces between FU and BSA were mainly hydrogen bond and Vander Waals force. The distance between FU and the tryptophan residue in BSA was 1.59 nm, and there was one binding site between FU and BSA. The UV/Vis and CD spectra of BSA in presence of FU showed that the binding of FU led to the conformation change of the protein.

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